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Factors Determining Degree of Inflation in Intratracheally Fixed Rat Lungs

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16. ABSTRACT

The total lung capacity (TLC) of rats was measured in vivo and was compared to the displacement volume of the lungs following intratracheal fixation with glutaraldehyde or formaldehyde solution. When glutaraldehyde was used the speed of infusion of the fixative was an important factor in the final degree of lung inflation achieved. With a low rate of fixative infusion and a final pressure of 20 cm of fixative the glutaraldehyde-fixed lungs inflated to 55% TLC. With a high initial flow of glutaraldehyde and a final pressure of 20 cm of fixative the lungs inflated to 84% TLC. Fixation of lungs inside the intact chest wall was found to result in a higher degree of inflation. With a reservoir height of 20 cm and a low rate of fixative infusion lungs fixed in situ reached 74% TLC, whereas lungs fixed in situ, but from animals that have been exsanguinated prior to fixation, inflated to only 58% TLC. This suggests that the volume of the blood in the lungs prior to infusion of glutaraldehyde influences the degree of inflation achieved. Formaldehyde-fixed lungs required 72 h to be completely fixed and they were inflated to 90% TLC when a reservoir height of 20 cm was used. Because of the slow rate of fixation using with formaldehyde solution the rate of infusion was found not to limit the degree of inflation that could be achieved.

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Factors determining degree of inflation in intratracheally fixed rat lungs

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HAYATDAVOUDI, GHOLAMHOSSAIN, JAMES D. CRAPO, FRED J. MILLER, AND JOHN J. O'NEIL. *Factors determining degree of inflation in intratracheally fixed rat lungs.* J. Appl. Physiol.: Respirat. Environ. Exercise Physiol. 48(2): 389–393, 1980.—The total lung capacity (TLC) of rats was measured in vivo and was compared to the displacement volume of the lungs following intratracheal fixation with glutaraldehyde or formaldehyde solution. When glutaraldehyde was used the speed of infusion of the fixative was an important factor in the final degree of lung inflation achieved. With a low rate of fixative infusion and a final pressure of 20 cm of fixative the glutaraldehyde-fixed lungs inflated to 55% TLC. With a high initial flow of glutaraldehyde and a final pressure of 20 cm of fixative the lungs inflated to 84% TLC. Fixation of lungs inside the intact chest wall was found to result in a higher degree of inflation. With a reservoir height of 20 cm and a low rate of fixative infusion lungs fixed in situ reached 74% TLC, whereas lungs fixed in situ, but from animals that have been exsanguinated prior to fixation, inflated to only 58% TLC. This suggests that the volume of the blood in the lungs prior to infusion of glutaraldehyde influences the degree of inflation achieved. Formaldehyde-fixed lungs required 72 h to be completely fixed and they were inflated to 90% TLC when a reservoir height of 20 cm was used. Because of the slow rate of fixation using with formaldehyde solution the rate of infusion was found not to limit the degree of inflation that could be achieved.

total lung capacity; displacement lung volume; glutaraldehyde; formaldehyde solution; inflation fixed lung volume

A COMMON METHOD of preparing the lungs of small animals for electron microscopic or morphometric studies is to instill the fixative through the trachea at hydrostatic pressures of 20–30 cmH_2O. The degree of inflation achieved in fixative-inflated lungs may have significant effects on subsequent morphometric measurements. Physiological studies have shown that a saline-filled lung is fully inflated at hydrostatic pressures of 6–7 cmH_2O (7); however, we noted that the fixed lung volumes reported in morphometric studies were usually significantly lower than the predicted total lung capacity (TLC) for animals of the same weight even when the lungs were fixed at hydrostatic pressures that exceeded 20 cmH_2O. For example, Burri et al. (2) reported that lungs from rats weighing 140 g had fixed lung volumes of 6.3 ml, of which approximately 2 ml were found to be tissue and slightly over 4 ml were found to be air in the alveoli and airways. The predicted TLC for 140-g rats is 7 ml (Takezawa, Miller, and O'Neil, unpublished data). Crapo et al. (4) studied rats weighing 360 g and reported a fixed lung volume of 10.8 ml, which represented an air volume of 9 ml. The predicted TLC for rats of this size is 13 ml. In both of these studies the air-space volume of fixed lungs was only slightly in excess of 60% of the predicted TLC. Because of this apparent discrepancy we conducted the following experiments to study the relationship of physiologically determined lung volumes with those achieved using fixatives to inflate the lungs.

MATERIALS AND METHODS

Animals. Specific pathogen-free CD strain, male rats (Charles River Breeding Laboratories, Portage, MI), weighing 160–400 g, were anesthetized with pentobarbital (50 mg/kg ip). A tracheostomy was performed on each rat and a 15-gauge Luer stub adapter (BD 8205) was placed in the trachea and tied securely.

Measurement of total lung capacity. We determined TLC of the anesthetized animals by gas-dilution techniques. Vital capacity (VC) was estimated as the volume of gas necessary to inflate the lungs from an airway pressure of 10 cmH_2O (residual volume) to an airway pressure of +25 cmH_2O (TLC). A volume of gas equivalent to the VC and containing 0.5% neon was then equilibrated with the gas in the lungs to determine TLC (10). All pressure measurements were made relative to atmospheric pressure, and the measurements of VC and TLC were done in triplicate. The mean value was calculated, corrected for tracheal air volume and converted to BTPS.

Fixatives. Glutaraldehyde fixatives were prepared in a sodium cacodylate buffer, pH 7.4, with a total buffer osmolarity of 170 mosM. The final osmolarity of our 2% glutaraldehyde fixative was 350 mosM. Formaldehyde solution (10% Formalin) was buffered to pH 7.4 using a potassium phosphate buffer.

Fixation techniques. Following determination of TLC the rat lungs were either fixed in situ or they were excised and fixed at different fixation pressures. Adequate fixation was defined as achieving sufficient tissue rigidity so that no fixative returned from the trachea when the fixative pressure was removed. Lungs were fixed in situ by opening the abdomen, puncturing both diaphragms to permit partial collapse of the lungs, and then rapidly

beginning an infusion of the fixative through the luer stub adapter that had been tied into the trachea. For all in situ fixatives, the height of the fixative reservoir above the midpoint of the animal's chest wall was 20 cm. The lungs to be fixed after excision were dissected free with the trachea and the tracheal cannula kept intact. To avoid possible effects of drying of the pleura on the fixed lung volumes, the fixation of all excised lungs was initiated within 90 s after opening the chest wall. After the initial inflation, the lungs were supported during the remainder of the fixation time by suspending them submerged in the fixative. The final fixative pressure was defined as the height of the fixative reservoir above the surface of the fixative in which the lungs were immersed. Lungs fixed with formaldehyde solution were held at their fixation pressure for 3 days. All fixations were done at 23–25°C. No significant leakage of fixative through the pulmonary vessels occurred with either fixative.

The flow rate at which the fixative was infused into the trachea was controlled by varying either the resistance of the tubing or th height of the fixative reservoir above the lungs.

The trachea and cannula were removed at the carina and weighed. The volume of the lungs was determined by clamping the airways at the carina and then using the volume-displacement method described by Scherle (8). To compare the fixed lung volume (which includes the volume of both lung tissue and the air spaces) with the TLC measured physiologically, we subtracted the volume of the lung tissue from the fixed lung volume and subtracted the volume of air in the trachea from the physiological TLC. The volume of tissue in the lung was determined by assuming it to have a specific gravity of 1.0, and therefore to be equal to the lung weight. Lung weights were estimated from a nomogram relating lung weight in rats (10).

Tissues were prepared for light microscopic and electron microscopic studies using standard techniques in our laboratory (4).

Statistical analyses. The effects of hydrostatic pressure and flow rate on excised fixed lung volumes were examined using two-way analysis of variance. Duncan's multiple-range test (3), a multiple-comparison procedure for pairwise testing of all treatment means, was employed to determine differences in fixation volume among lungs fixed in situ, in situ after exsanguination, and after excision. To more adequately control the experimentwise error, this test was used only when the univariate F test was significant (1).

RESULTS

We fixed excised lungs at five different pressures using either high-resistance (slow-flow) or low-resistance (high-flow) tubing between the reservoir and the lungs. The high-resistance tubing had a flow rate of 60 ml/min at 20 cmH_2O pressure when the system (including the Luer stub adapter) was not tied into a trachea. Two sets of low-resistance tubing were used. They produced flows of 125 and 160 ml/min at 20 cmH_2O pressure when the system was not tied into a trachea.

Figure 1 shows the relationship between hydrostatic pressure or flow rate and excised fixed lung volumes using 2% glutaraldehyde as the fixative. Three rat lungs were fixed at each pressure with the 125 ml/min system and five rat lungs were fixed at each pressure with the 160 ml/min system. Analysis of variance showed that these two low-resistance systems were not statistically different so that these data could be pooled.

When lungs were fixed with 2% glutaraldehyde at 20 cmH_2O of hydrostatic pressure using the high-resistance system, the air-space volume in the fixed lungs was only 55% of the TLC as measured previously by gas dilution. The rat lungs fixed at 20 cmH_2O pressure using the low-resistance system had an air-space volume of 84% of the previously determined TLC.

The rate at which glutaraldehyde is infused into a lung influences the degree of final inflation. The fixative pressure, the resistance of the tubing, and the resistance of the lung determine the rate of fixative flow. When the resistance in the lung itself becomes the dominant resistance, further reduction in the tubing resistance will not result in higher net flow rates. This is demonstrated in Fig. 2 where tubing resistance was varied by reducing the length of the tubing, and the final resistance was varied by using different sizes of small Luer stub adapters to represent the lung's resistance. Once a sufficiently high final resistance was reached (high gauge adapter) the flow rate was independent of the resistance of the tubing. This may explain the finding that reducing the resistance in our fixation apparatus to produce a tubing flow rate higher than 125 ml/min did not lead to any further increase in lung inflation.

We found that formaldehyde solution required 72 h to fix the lung to the point where there was no retraction (or recoil) of the tissue when the fixative pressure was

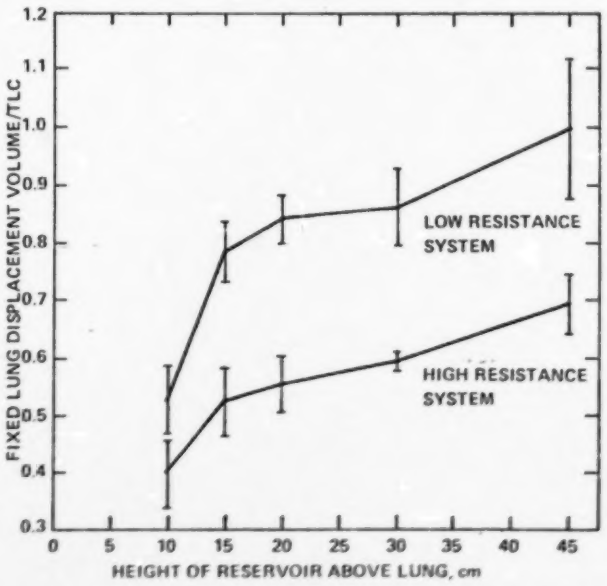

FIG. 1. Degree of inflation achieved in excised glutaraldehyde-fixed lungs at different hydrostatic pressures. *Top curve* shows this relationship of fixation volume to fixation pressure when a low-resistance (high-flow rate) system is used. For *bottom curve* a high-resistance (low-flow rate) system was used. Each point represents group means ± SD; $n = 8$.

temporarily released. This characteristic of slow fixation makes formaldehyde an undesirable fixative for electron microscopy owing to the loss of fine cellular and subcellular structural detail. When excised rat lungs were fixed with formaldehyde, the degree of inflation (Fig. 3) is primarily dependent on the hydrostatic pressure. Inflation with formaldehyde solution and a reservoir height of 20 cm resulted in a fixed lung air-space volume equivalent to 83% TLC. Further increases in pressure did not result in further increases in the fixed lung volume.

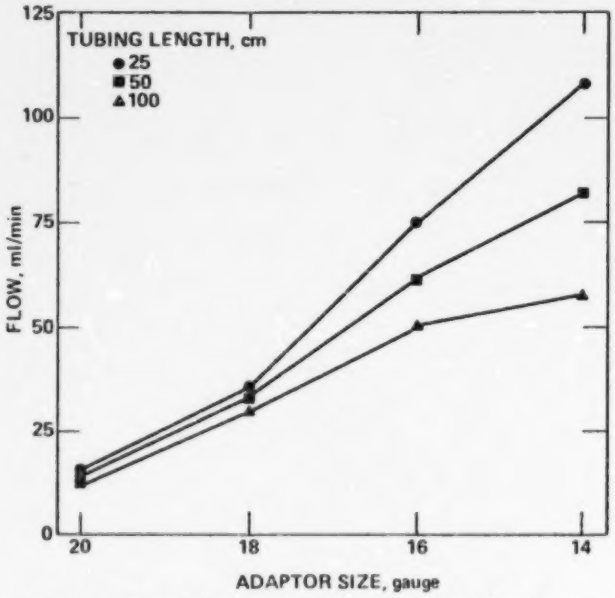

FIG. 2. Effects of changes in relative resistance of a fixation apparatus and flow rate of fixative achieved. Resistance was altered by using tubing of different lengths and by varying size of final Leur stub adapter.

We postulated that glutaraldehyde fixation was so rapid that the lung became partially rigid during infusion of the fixative. Thus slow infusions would result in low degrees of inflation. Formaldehyde being a slow-acting fixative would not be influenced by the rate of fixative infusion. To test this hypothesis we changed the concentration of glutaraldehyde to alter its speed of fixation. Figure 4 shows that there is an inverse relationship between the degree of inflation and the concentration of glutaraldehyde. Although high degrees of lung inflation can be achieved using 0.25% glutaraldehyde the quality of fixation as assessed by electron microscopy was poor when the glutaraldehyde concentration was less than 1%.

We also measured the fixation volume of excised lungs in which fixation was started from different lung volumes (Fig. 5). The lungs of four animals were degassed by first allowing anesthetized animals to breathe 100% O_2 for 2 min. The trachea was then occluded and the lungs rapidly decreased in size as the oxygen was absorbed. When the lungs appeared free from any residual gas, they were removed for fixation. The lungs of another four rats were dissected out and fixed at their excised residual volume. In a third and fourth group of rats, a volume of air equivalent to 25 and 50% of the measured VC was injected into the lungs from the excised residual volume and the infusion of fixative was commenced at this degree of inflation. All of these lungs were fixed using a high-flow (low-resistance) system. There was no statistically significant difference between the fixed volume of the lungs degassed prior to inflation and those partially inflated prior to fixation.

When lungs were fixed in situ using 2% glutaraldehyde with the reservoir at 20 cm and a low-flow (high-resistance) system, the lungs were inflated to 74% of their TLC (Fig. 6). This is significantly higher than the 55% inflation

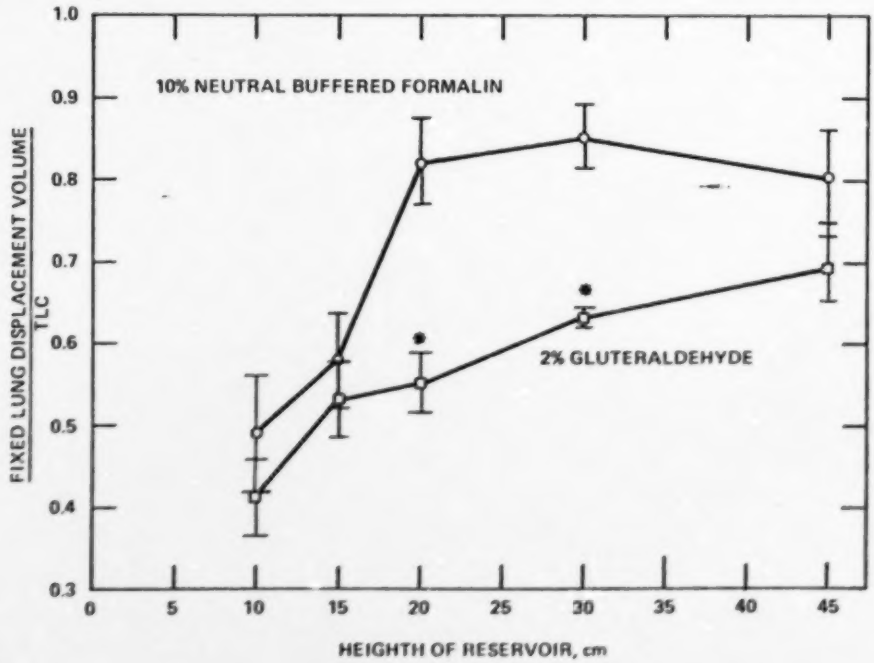

FIG. 3. Degree of lung inflation achieved in excised lungs fixed at pressure from 10–45 cm of fixative pressure using 10% neutral buffered formaldehyde solution or 2% glutaraldehyde. Data are means ± SD; $n = 3$. *Asterisk* indicates that difference in degree of inflation achieved by two fixatives was statistically significant ($P < 0.05$).

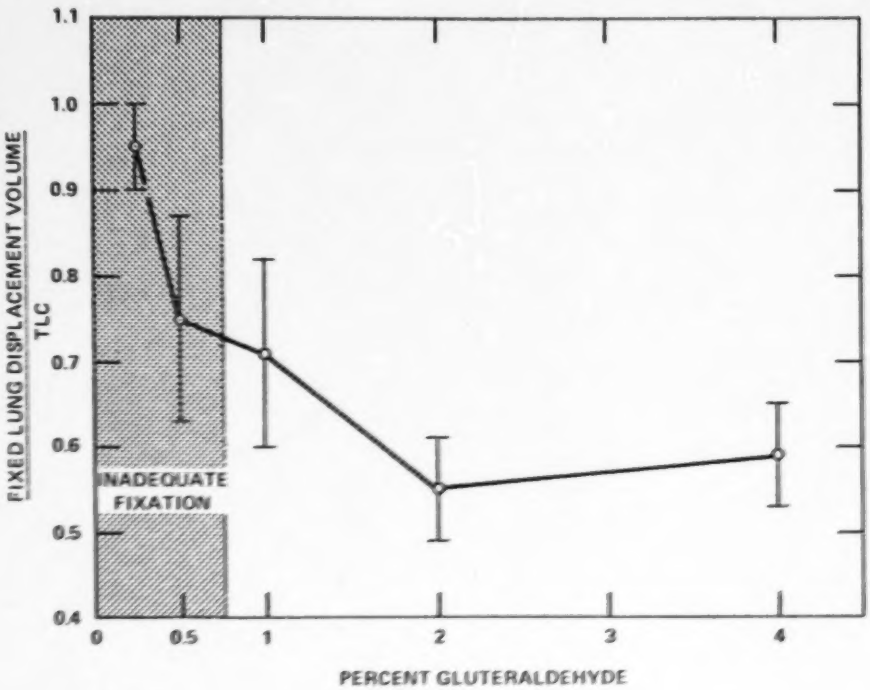

FIG. 4. Degree of lung inflation achieved using different concentrations of glutaraldehyde. All fixations were done on excised rat lungs using 20 cm of fixative pressure intratracheally with a high-resistance (low-flow rate) fixative system. Electron microscopic evaluation revealed inadequate fixation of lung inflated with concentrations of glutaraldehyde less than 1%. Data are means ± SD; $n = 3$.

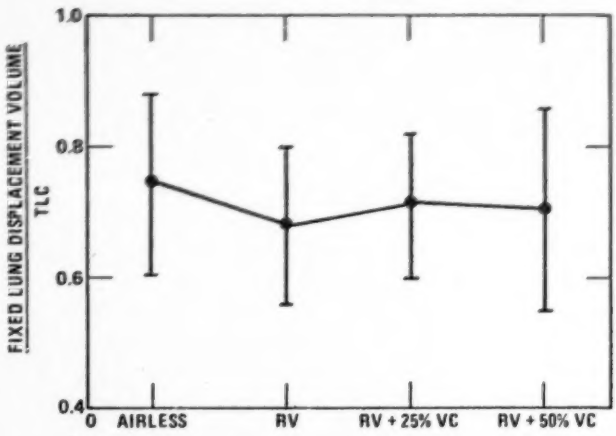

FIG. 5. Degree of lung inflation achieved when excised lungs are fixed intratracheally with 2% glutaraldehyde starting from different initial lung volumes. Data are means ± SD; $n = 4$.

obtained when the lungs were fixed outside the chest. When a high-flow (low-resistance) system was used to compare the fixation of in situ and excised lungs no significant difference in the degree of inflation was found (Fig. 6). We postulated that the blood contained in lung in situ may influence the degree of lung inflation achieved with fixatives. We evaluated this by cutting the abdominal aorta in a group of rats to exsanguinate them prior to fixing their lungs in situ. Exsanguination resulted in a statistically significant ($P < 0.05$) drop in the fixed lung air-space volume when compared to in situ fixed lungs under otherwise identical conditions of fixation (this was true using both the high-flow and low-flow fixation systems) (Fig. 6).

DISCUSSION

The fixed lung volume is essential for morphometric determination of tissue densities, surface areas, and volumes of air and tissue compartments of the lung. Any change in lung volume may result in changes in surface area, alveolar and capillary volumes, mean alveolar chord length, or in the thickness of tissue compartments.

Many studies of lung tissue have been conducted using glutaraldehyde fixation at 20–30 cmH_2O pressure (2, 4–6). We are unaware of any study to date in which the fixation flow rate has been reported. Most authors have appeared to assume that a constant (or maximal) degree of inflation occurs when the fixative is infused at a constant pressure of 20 cmH_2O or more. Figure 1 shows that this assumption is clearly incorrect if glutaraldehyde is used as the fixative. One can achieve maximal lung inflation using glutaraldehyde if attention is given to maintaining very high fixative flows at the initiation of fixation. Maximal lung inflation can be more easily achieved using formaldehyde as the fixative, but maintaining the fixative inflation pressure for 72 h is required. Fixation using formaldehyde solution at a fixative pressure of 25 cm for only 24 h has been reported to give a displacement lung volume which is only 61% of the plethysmographic TLC_{25} (9).

We were surprised to find that significantly higher degrees of lung inflation could be achieved by fixing the lungs in situ. We identified between the lungs fixed in situ and those excised before fixation two major differences that might have explained the different degrees of inflation achieved. One was that the in situ lungs contained a greater volume of blood (the heart was still beating at the time fixation was started). A second was that in the process of excising the lungs the manipulation may have reduced their "excised residual volume" caus-

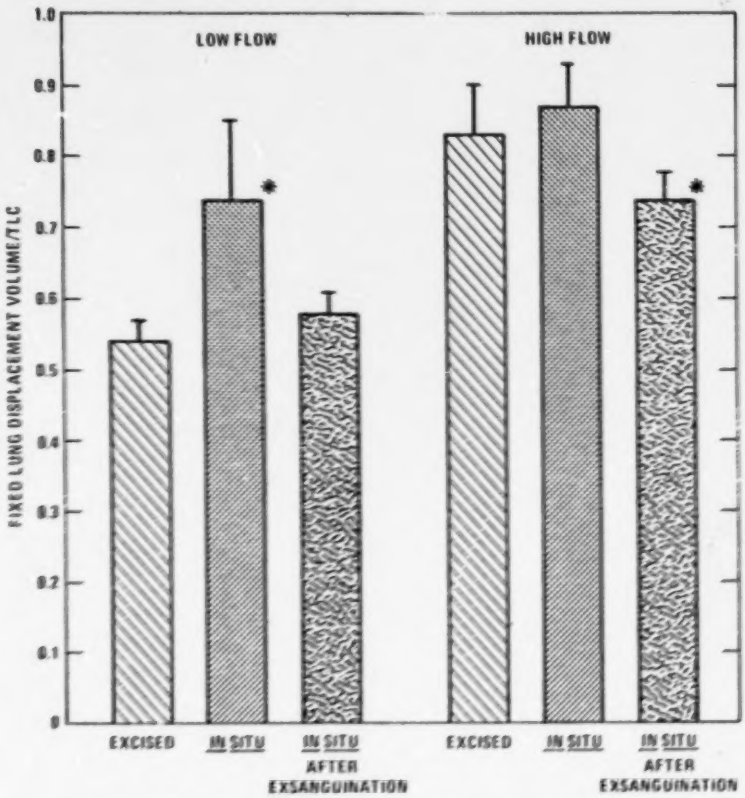

FIG. 6. Changes in relative degree of inflation achieved when lungs are excised prior to initiating intratracheal glutaraldehyde fixation. Rat lungs were fixed in situ with chest wall intact, in situ after exsanguination by transection of the abdominal aorta, and after excision from the animal. Both a high-resistance (low-flow) system and a low-resistance (high-flow) system were used for the fixations. Final fixation pressure was 20 cm of fixative. Data are means ± SD; $n = 5$. *Bars* marked by an *asterisk* are statistically different ($P < 0.05$) from other two groups fixed at same flow.

ing them to begin the fixation process at a lower lung volume. We tested these two postulates, and found that the lung volume at the initiation of fixation did not affect the final fixed lung volume but that the presence of circulating blood in the lung at the time fixation was initiated does increase the final fixed lung volume (Fig. 6).

The difference in the degrees of inflation achieved in in situ and in excised lungs could be reduced by increasing the flow rate at which the fixative was instilled (Fig. 6). At the higher lung volumes achieved with higher fixative flow rates the compliance of the chest wall may become a factor. The compliance of the chest wall in the rat is high, but at high lung volumes it may be enough to partially restrict the degree of inflation achieved in situ.

We conclude that the volume of intratracheally fixed lungs depends on *1*) type of fixative; *2*) initial flow rates of the fixative; *3*) final pressure of the fixative; *4*) whether the lungs are fixed in situ or after excision; and *5*) conditions that may affect the volume and/or pressure of blood in the lung at initiation of fixation. Studies of fixative-inflated lungs should report these variables. Ideally the degree of lung inflation achieved using fixatives should be determined by measuring both the total lung capacity using physiological techniques and the displacement volume of the fixed lung. Most previous morphometric studies appear to have been done at lung volumes equivalent to 50–70% TLC. This is a more physiological degree of lung inflation than TLC and may be the most appropriate range in which morphometric and morphologic studies should be conducted. However, the degree of inflation achieved by 20 cm of fixative pressure with glutaraldehyde will be highly variable depending on other parameters as described above. Thus measured values such as surface area, tissue thickness, and tissue densities may not be fully comparable between different studies.

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